

REPORT DOCUMENTATION PAGE

AD-A256 415

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 27 Jan 92		3. ANNUAL Technical Feb 90 - Feb 92	
4. TITLE AND SUBTITLE The Effect of Hyperbaric Oxygen and Pentoxifylline on the Rate of Neovascularization in Mice.				5. FUNDING NUMBERS AFOSR-89-0543 PE 61102F PR 2312 TA A5	
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10. SUPPLEMENTARY NOTES None					
11. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
12. ABSTRACT (Maximum 200 words) A polyvinyl alcohol sponge was implanted in mouse subcutaneous tissue to investigate two treatments [intermittent hyperoxia (100% oxygen for 90 mins twice a day at 250 kPa) and epidermal growth factor (EGF)] which may modulate fibroblast infiltration. Two conditions were established for treatment: exposure of animals to chronic hypoxia (12% oxygen for 23 hr/day), simulating low oxygen tensions in problem wounds, and normoxia (21% oxygen). In experiments evaluating EGF, sponges were implanted whose core contained EGF covered with a slow release polymer, the other group with placebo. Sponges were harvested at 15, 25, and 32 days after implantation. The area of the disc infiltrated by fibroblasts was measured by planimetry. After 32 days exposure to hypoxic conditions (7 days before sponge implantation and 25 days after) EGF slightly increased (NS) the area of fibroblast infiltration compared to placebo under both hypoxic and normoxic conditions. No significant differences were observed between the hypoxically conditioned groups and normoxic controls. Neither chronic hypoxia alone nor chronic hypoxia with intermittent hyperbaric oxygen administered 21-32 days after disc implantation affected the area of fibroblast infiltration. EGF significantly increased the area of					
13. SUBJECT TERMS Fibroblast-Hyperbaric Oxygen-Hypoxia-Polyvinyl Alcohol Sponge-Wound				14. NUMBER OF PAGES	
15. SECURITY CLASSIFICATION OF REPORT Unclassified				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF THIS PAGE SAR		18. SECURITY CLASSIFICATION OF ABSTRACT SAR		19. LIMITATION OF ABSTRACT SAR	

Abstract

13. A polyvinyl alcohol sponge was implanted in mouse subcutaneous tissue to investigate two treatments [intermittent hyperoxia (100% oxygen for 90 mins twice a day at 250 kPa) and epidermal growth factor (EGF)] which may modulate fibroblast infiltration. Two conditions were established for treatment: exposure of animals to chronic hypoxia (12% oxygen for 23 hr/day), simulating low oxygen tensions in problem wounds, and normoxia (21% oxygen). In experiments evaluating EGF, sponges were implanted whose core contained EGF covered with a slow release polymer, the other group with placebo. Sponges were harvested at 15, 25, or 32 days after implantation. The area of the disc infiltrated by fibroblasts was measured by planimetry. After 32 days exposure to hypoxic conditions (7 days before sponge implantation and 25 days after) EGF slightly increased (NS) the area of fibroblast infiltration compared to placebo under both hypoxic and normoxic conditions. No significant differences were observed between the hypoxically conditioned groups and normoxic controls. Neither chronic hypoxia alone nor chronic hypoxia with intermittent hyperbaric oxygen administered 21-32 days after disc implantation affected the area of fibroblast infiltration. EGF significantly increased the area of the fibrous capsule around small PVA sponges after 15 days under normoxic conditions.

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1. Objectives

This study examined the effects of hypoxic conditioning, hyperbaric oxygen treatments, and epidermal growth factor administration on the rate of fibroblast infiltration into a polyvinyl alcohol (PVA) sponge model of wound healing using mice. The specific objectives were as follows:

- a) Determine the effect of hypoxic conditioning on fibroblast infiltration into the PVA sponge model.
- b) Determine whether hyperbaric oxygen therapy altered the effects of hypoxic conditioning on fibroblast infiltration.
- c. Determine whether the effects of epidermal growth factor (EGF) on fibroblast infiltration would be altered by hypoxic conditioning.

It was the original intention of the protocol to directly measure the extent of neovascularization into the PVA sponge. It was decided that measuring fibroblast infiltration into the sponge and using it as an indirect measure of neovascularization was a more accurate and objective quantification of response of the wound healing model than actually measuring the extent of infiltration of individual vessels. Also the area of fibroblast infiltration was felt to be a better experimental analogue of granulation tissue in a wound and thus more relevant to wound healing than measurement of the infiltration of individual vessels.

Originally we planned to use the microcirculatory drug, pentoxifylline, to examine interactions with hypoxic conditioning and hyperbaric oxygen therapy with mice. Because of recent experimental findings we decided epidermal growth factor was a more promising agent for wound healing treatments and we used it in place of pentoxifylline to examine interactions of wound healing with hypoxic conditioning and hyperbaric oxygen treatments.

2. Findings

A comprehensive statement of the research can be found in the appendices. The major findings of this study are summarized as follows:

- 1) Conditioning of mice to a hypoxic environment did not effect the extent of fibroblast infiltration into the polyvinyl alcohol sponges.
- 2) Hyperbaric oxygen exposures did not alter fibroblast infiltration in hypoxically conditioned animals.
- 3) Epidermal growth factor slightly increased the extent of fibroblast infiltration in hypoxically conditioned animals and controls.
- 4) Epidermal growth factor significantly increased the area of fibrous capsule around the PVA sponge.

5) Hypoxic conditioning reduced subcutaneous tissue oxygen levels. Whether mice were acutely exposed or chronically conditioned to hypoxia subcutaneous tissue oxygen levels were approximately half that of normoxic controls. No difference was seen between subcutaneous tissue oxygen tensions of mice exposed to the hypoxia for 10 min compared to 10 days.

3. Presentations and Publications.

17 April 1991. Side presentation given to the professional staff of the Armed Forces Institute of Pathology.

2 October 1991. Publication of abstract (Appendix A) and poster presentation delivered to a meeting of the American Physiological Society held in San Antonio, TX. Criswell DW, Mehm WJ. Effect of hyperoxia and epidermal growth factor on fibroblast infiltration and neovascularization. *Physiologist* 1991;34:256.

27 January 1992. Abstract (Appendix B) submitted for a slide presentation to be delivered at the meeting of the Undersea and Hyperbaric Medical Society 23-27 Jun 1992 at Bethesda, MD.

27 January 1992. Research paper (Appendix C) in final preparation for submission to the *Journal of Hyperbaric Medicine*. Two additional papers

are in preparation for submission to journals. One will be the work presented in the abstract in Appendix B concerning tissue oxygen measurements and will be submitted to the "Journal of Hyperbaric Medicine". Another will concern the effect of hypoxic conditioning and epidermal growth factor on fibroblast infiltration into PVA sponges in rats and will be submitted to the journal "Wounds".

4. Appendices.

Appendix A - Abstract of poster presentation delivered at the 1991 meeting of the American Physiological Society and published in the Physiologist 1991;34:256.

Appendix B - Abstract submitted for the 1992 meeting of the Undersea and Hyperbaric Medical Society 23-27 June 1992 in Bethesda, MD.

Appendix C - Research paper prepared for submission to the Journal of Hyperbaric Medicine.

APPENDIX A

1991 APS CONFERENCE
ABSTRACT FORM

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APS OFFICE BY FRIDAY,
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Membership Services Department
American Physiological Society
9650 Rockville Pike
Bethesda, Maryland 20814

MAILING ADDRESS OF FIRST AUTHOR

(Please print in black ink or type. Provide full name rather than initials.)

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EFFECT OF HYPEROXIA AND EPIDERMAL GROWTH FACTOR ON FIBROBLAST INFILTRATION AND NEOVASCULARIZATION. D.W. Criswell* and W.J. Mehm. Armed Forces Institute of Pathology, Washington, DC 20306.

A polyvinyl alcohol sponge disc (13mm diameter) was implanted in mouse subcutaneous tissue to investigate two treatments [epidermal growth factor (EGF), and intermittent hyperoxia (100% oxygen for 90 mins twice a day at 250 kPa)] which may modulate neovascularization. Two conditions were established for treatment: exposure of animals to chronic hypoxia (12% oxygen for 23 hrs/day), simulating low oxygen tensions in problem wounds, and normoxia (21% oxygen). In experiments evaluating EGF, discs were implanted whose core contained EGF covered with a slow release polymer, the other group with placebo. Discs were harvested at 14, 25, or 32 days after implantation. The area of the disc infiltrated by fibroblasts was measured by planimetry and used as an indirect measure of neovascularization. When EGF (20 µg) was administered for 14 days under hypoxic and normoxic conditions no effect was seen on neovascularization. However, after 25 days under normoxic conditions EGF slightly increased (NS) neovascularization compared to placebo (24.8 mm², n=7, vs. 18.4 mm², n=10). Exposure of chronically hypoxic and normoxic animals to intermittent hyperoxia between 21-32 days of disc implantation without EGF did not affect of neovascularization. EGF may have more potential than hyperoxia in promoting neovascularization.

All compounds that are designated by code or initial letters must be identified adequately in the abstract, e.g., MJ-1999: 4-(2-isopropylamino-1-hydroxyethyl) methanesulfonanilide hydrochloride.

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☐ Submitted for special CRS Student Award

William J. Mehm, Maj, USAF, BSC

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William J. Mehm

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APPENDIX B

1st Choice: # 7 Title Physiol Biochem Effect
2nd Choice: # 6 Title HBO Therapy

PRESENTATION PREFERENCE (scientific sessions only)
Preferred choice (CHECK ONE ONLY)

☒ Slide presentation ☐ Poster presentation
☐ Either

1992 UNDERSEA AND HYPERBARIC MEDICAL SOCIETY
ANNUAL SCIENTIFIC MEETING ABSTRACT FORM

SUBCUTANEOUS OXYGEN TENSION CHANGES WITH ALTERATION OF AMBIENT OXYGEN PRESSURE IN MICE. D.W. Criswell and W.J. Mehm. Armed Forces Institute of Pathology, Washington DC. 20306-6000

The objective of this study was to ascertain changes in subcutaneous tissue oxygen tensions in mice used in an experimental model of wound healing. We altered tissue oxygen tensions by varying the ambient oxygen pressure to which the mice were exposed. Anesthetized mice were exposed to 12%, 21%, and 100% oxygen at an ambient pressure of 100 kPa and to 100% oxygen at 250 kPa. Oxygen tension measurements were made with a Clark type combination oxygen electrode encased in a syringe needle. The syringe electrode was inserted through the skin into the subcutaneous tissue in the back of the mouse at the site the polyvinyl alcohol sponge was implanted in the experimental model. Readings were recorded 10 min after transfer from a normoxic to a experimental environment. Subcutaneous oxygen tension rose with increasing ambient oxygen pressure as listed below.

% Oxygen	Ambient Pressure (kPa)	Tissue Oxygen Tensions (mmHg)
12	100	19.0 \pm 1.4
21	100	34.8 \pm 1.8
100	100	93.8 \pm 9.8
100	250	529.0 \pm 75.1

To determine if conditioning mice to a hypoxic environment (12% oxygen) resulted in any difference in tissue oxygen tension compared to acute exposure, we exposed mice to 12% oxygen for 12 days. No adaptive response was seen to the conditioning since tissue oxygen levels were not significantly different between mice conditioned to hypoxia (17.4 \pm 1.7 mmHg) compared to the animals acutely exposed to the hypoxia (19.0 \pm 1.4 mmHg). Using a simple experimental technique we quantified the oxygen tensions achieved by the alteration of oxygen pressures.

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APPENDIX C

Effect of Hyperoxia and Epidermal Growth Factor on Fibroblast
Infiltration into Polyvinyl Alcohol Sponges in Mice

D.W. Criswell, Maj, USAF, BSC and
W.J. Mehm, Maj, USAF, BSC

Division of Altitude and Hyperbaric Physiology
Armed Forces Institute of Pathology
Washington, DC 20306-6000

Running Head: Hyperoxia, EGF, and Fibroblast Infiltration

Please address correspondence to Major W. J. Mehm, CPW-P, Armed
Forces Institute of Pathology, Washington, DC 20306-6000.

We wish to thank David Nelson and Bernard Wilson for their technical assistance with this project. The opinions or assertions are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Air Force or of the Department of Defense. This work was funded by a grant from the Air Force Office of Scientific Research #89-0543. The experiments reported herein were conducted according to the principles described in "Guide for the Care and use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, U.S. Department of Health and Human Services Publication No. (NIH) 85-23.

Introduction

Tissue damage often results in discontinuity of the body's integument. The body must try to repair the discontinuity of a wound by utilizing existing tissue (wound contraction) or forming new tissue (scar formation). Both oxygen (1) and growth factors (2) play a vital role in the wound healing process. Oxygen is specifically required by the fibroblast for the function of dioxygenase enzymes (3) which hydroxylate proline and lysine residues before extracellular secretion of the collagen molecule. These collagen molecules form scar tissue, which bridges the wound, and also form fibers which provide structural support for blood vessel ingrowth into the avascular wound. Growth factors are secreted by activated macrophages, platelets and other cells in connective tissue, and are responsible for increasing the proliferation of fibroblasts and endothelial cells in the area of damaged tissue (4). These fibroblasts and endothelial cells form the collagen and provide the vascularity for the tissue ingrowth into the avascular area.

Animal experiments show that reduction of ambient oxygen decreases wound closure (5) and collagen deposition in polyvinyl alcohol (PVA) sponges (6). These experiments also demonstrate an increase of wound closure rate and collagen deposition with increased ambient oxygen pressures. Intermittent hyperbaric oxygen administration increases wound closure rate in hypoxic wounds but not in normoxic wounds (7). Growth factor administration has been shown to increase collagen deposition in

PVA sponges in animals (8) and to promote healing in human wounds (9).

Wound healing may be impaired because of reduced blood perfusion (ischemia), or tissue hypoxia. These phenomena are commonly seen in humans with tissue perfusion problems, such as microvascular disease (10). The lack of oxygen at the wound site limits healing because of reduced collagen deposition necessary for the formation of scar tissue and new blood vessels (11,12). Ischemia itself may be responsible for tissue hypoxia at the wound site and may also limit the supply of nutrients and inflammatory cells to the wound area, thus impairing tissue repair. Therapeutic intervention by elevation of the inspired oxygen tension, or the exogenous administration of growth factors attempts to overcome the deficiencies responsible for the non-healing wound.

Elevated inspired oxygen tensions and growth factors attempt to promote the healing of the wound by different mechanisms. Hyperoxic treatments attempt to overcome the detrimental effects of tissue hypoxia resulting from impaired blood perfusion by increasing the extracellular production of collagen and thus collagen deposition into the wound. Growth factor administration attempts to use endogenous chemical mediators to stimulate healing by promoting the growth of scar and vascular tissue. Despite research supporting the value of these interventions, their relative efficacy in treating non-healing wounds has not been precisely defined.

Although oxygen and growth factors have been shown to alter neovascularization and collagen deposition in wound healing, their

interactive effects have not been examined. The purpose of this study is to examine fibroblast infiltration in PVA sponges in mice exposed to a reduced ambient oxygen pressure, simulating the low tissue oxygen levels characteristically found in non-healing wounds. The separate and combined effects of hypoxic exposures, intermittent hyperbaric oxygen exposures, and epidermal growth factor (EGF) on fibroblast infiltration will be compared.

Materials and Methods

Animals: Female 25-30 gm mice (CF1, Harlan Sprague Dawley Co, Ind, MN) were used in all experiments to allow the placement of multiple animals in a single cage without cannibalism. The mice were given food and water ad libitum and maintained on a 12 hr light/12 hr dark cycle. During the experiment the animals were kept in 29 x 19 x 13 cm animal cages which were placed in ventilated clear plastic boxes for control of atmospheric gases. Mice were anesthetized prior to both surgery or oxygen tension measurement using a subcutaneous injection of ketamine (50 mg/kg) and xylazine (20 mg/kg). Euthanasia was preceded by anesthetic injection and accomplished by cervical dislocation.

Experimental Model: Polyvinyl alcohol sponges were prepared in our laboratory and implanted in animals according to the method of Fajardo (13) as briefly described in the following section. A 2 cm surgical incision is made in the left hindquarter of the mouse. A single PVA

sponge disc is surgically inserted into the subcutaneous tissue of each mouse contralateral to the surgical incision. The sponge is 13 mm in diameter by 1 mm thick and is covered on both sides of the disc with a millipore filter to allow diffusion of extracellular fluid into the edges of the disc without cellular infiltration. The sponge remains in the animal for the duration of the experiment, is then removed surgically and fixed in formalin. The sponge is sectioned, stained with hematoxylin and eosin, and examined by morphometry. This area of infiltration of the sponge by fibroblasts is measured by computerized planimetry (TAS Plus, Image Analysis System, Leica, Inc, Rockaway, New Jersey).

Tissue Oxygen Measurement: Subcutaneous tissue oxygen tension measurements were sampled to confirm that tissue oxygen tensions were altered by exposing mice to hypoxic or hyperoxic environments. An implantable Clark type combination PO₂ needle electrode with Ag/AgCl reference electrode (Diamond Electro-Tech Inc, Ann Arbor, Michigan) was used to measure tissue oxygen tension. Measurements of oxygen tension derived from electrical potentials from the electrode were read with a Chemical Microsensor System (Diamond General Corp, Ann Arbor, Michigan). These electrodes are encased in a syringe which is inserted through the skin into the subcutaneous tissue in the back of an anesthetized mouse. Readings of oxygen tensions were taken 10 min after electrode insertion or the animal being placed in a different atmosphere. Readings were obtained from 5 animals in each experimental group when exposed to their designated gas environments.

Statistical Analysis: Results were analyzed by the two way Analysis of Variance. Probability level for statistical significance was set at $p \leq 0.05$.

Experimental Design: Animals were divided into three experiments as illustrated in the flow diagram in Fig 1.

Experiment 1 - Effect of intermittent hyperbaric oxygen exposures on hypoxic mice: This experiment investigated the effects of intermittent hyperbaric oxygen therapy on fibroblast infiltration into PVA sponges in hypoxic mice compared to normoxic controls. Following sponge implantation, mice were maintained in normoxic conditions (21% oxygen) for 21 days prior to the exposure to the hypoxic (12% oxygen) condition for 11 days. This allowed the inflammatory reaction of the mice to encapsulate and vascularize the sponge, prior to the ingrowth of fibroblastic tissue into the sponge. Three experimental treatments within Experiment 1 were provided (Fig. 1):

- 1) Hypoxia with intermittent hyperbaric oxygen exposures,
- 2) Hypoxia alone with sham hyperbaric dives,
- 3) Normoxia with sham hyperbaric dives.

Animals were sacrificed the day following the last hyperbaric chamber dive, or 32 days post sponge implantation.

Hypoxic Conditioning Exposures: To lower tissue oxygen tensions to simulate the low oxygen tensions in non-healing wounds, mice were placed in a hypoxic environment. Animals were exposed to a hypoxic atmosphere of 12% oxygen and 87% nitrogen (other trace atmospheric gases 1%) which was

produced by blending streams of nitrogen and compressed air with flowmeters. This blend of oxygen deficient (hypoxic) air was fed (1 L/min) into a ventilated clear atmospheric box (18 x 18 x 18 cm) containing the mice in their standard animal cages. Animals in the hypoxic conditioning groups were exposed to normoxic ambient air only for routine care and experimental manipulation, not exceeding 0.5 hr/day. Gas composition in these atmospheric boxes was measured daily to ensure carbon dioxide levels did not exceed 1%. Monitoring of the oxygen pressures in the box revealed readings of 78-83 mmHg (slightly lower than the 91 mmHg calculated for 12% oxygen due to metabolic consumption of oxygen by the mice).

Intermittent hyperbaric oxygen exposures: Hyperoxic exposures were administered in specially constructed cylindrical polyvinyl chloride hyperbaric chambers (0.33 x 0.75 M). Medical grade oxygen was used to pressurize the chambers. Both ascent and descent were performed at a rate of 50 kPa/min which is a rate comparable to those used for human exposures in clinical treatments (14). No untoward effects on the animals were observed during or following the hyperbaric exposures. Chambers were ventilated continuously at a rate of 2 L/min. Animals receiving sham exposures (controls) were placed in chambers ventilated with the gas to which they were chronically exposed (hypoxic or normoxic air). The hyperbaric exposures were administered at a pressure of 250 kPa for a duration of 90 min twice daily (b.i.d.) over an initial 5 day period followed by a 2 day break, and then for an additional 4 days of b.i.d.

hyperbaric treatments before sacrifice (the 2 day break was designed to simulate the weekend treatment breaks experienced during human hyperbaric treatments). Mice received a total of 18 dives in a 12 day period.

Experiment 2 - EGF's effect in hypoxically conditioned mice: The period of hypoxic conditioning was increased to determine if a longer hypoxic exposure than the one in Experiment 1 would affect fibroblast infiltration in hypoxic mice. Mice were exposed to a hypoxic or a normoxic environment for 7 days prior to and 25 days post sponge implantation to determine the effect of prolonged exposure to the hypoxic environment on fibroblast infiltration (Fig 1). Hypoxic exposures were accomplished as described in experiment 1. We examined the effect of EGF in the wounds by implanting half of the mice in each group with EGF impregnated PVA sponges. The EGF was incorporated into the sponge in a slow release polymer and this was released throughout the course of the sponge implantation. There were four experimental groups:

- 1) Normoxia with EGF,
- 2) Normoxia with placebo,
- 3) Hypoxia with EGF,
- 4) Hypoxia with placebo.

Epidermal Growth Factor: Synthetically produced human EGF, fragment 20-31, was purchased from Sigma Chemical Corporation, St. Louis, MO (#E-9384). This EGF possesses the same biological activity as EGF extracted from salivary glands (15). Prior to incorporation in the sponge implant the EGF was reconstituted to a concentration of 20 ug/ml in

phosphate buffered saline. Epidermal growth factor was incorporated into a 1.5 mm diameter central core section of the sponge. Twenty ng of EGF dissolved in phosphate buffered saline, was absorbed into the pellet, allowed to dry, and then the pellet was coated with 10 ul of a 5% solution of the slow release polymer, ethylene-vinyl acetate (Elvax, DuPont). Controls (placebo treatment) had 20 ul phosphate buffered saline absorbed into the pellet, allowed to dry, and then coated with 5% Elvax.

Experiment 3 - EGF and fibrous capsule area: This experiment examined the ability of the growth factor to affect the size of the fibrous capsule surrounding the sponge. To make it easier to separate the fibrous tissue attached to the sponge from the surrounding connective tissue, small sponges were used. Polyvinyl alcohol sponges were prepared in an identical manner to the ones described above except they were approximately 1/3 the diameter (5 mm in diameter) of those used in the previous experiment. Twenty ng of the EGF was absorbed into the entire sponge. The sponge was then implanted in mice maintained in a normoxic atmosphere for 15 days. Both the area of fibroblast infiltration and the area of fibrous encapsulation around the sponge were measured by planimetry.

Results

Tissue oxygen tension measurement: Subcutaneous tissue oxygen tension rose in mice exposed to high ambient oxygen pressures and fell in

mice exposed to low ambient oxygen pressures. Ten min after mice were taken from normoxic conditions and placed in a hypoxic environment the tissue oxygen tensions fell from a mean of 34.8 ± 1.7 mmHg to 17.4 ± 1.7 mmHg. When mice were conditioned to a hypoxic environment for 12 days they showed a tissue oxygen tension of 35.8 ± 2.0 mmHg 10 min after exposure to ambient air and the tension fell to 19.0 ± 1.4 mmHg 10 min after being returned back to the hypoxic environment. Mice transferred to a 100% oxygen environment showed their tissue oxygen tensions to rise from 34.6 ± 1.4 to 93.8 ± 9.8 mmHg 10 min after transfer to 100% oxygen.

Cellular infiltration of the sponge: Fibroblasts and blood vessels infiltrate the complex trabeculae of the sponge after implantation in experimental animals. Inflammatory cells, leukocytes and macrophages, advance through the complex trabeculae of the sponge toward the center and are followed by an ingrowth of tissue consisting of fibroblasts and collagen fibers. This fibroblastic tissue infiltration forms a ring of tissue which penetrates through the free edges of the sponge toward the center of the sponge. This ring of tissue advances toward the center of the sponge with increasing time after implantation in the experimental animal. The ring of fibroblastic tissue infiltrating the sponge is the region measured by planimetry. Surrounding the sponge is a capsule of fibrous tissue containing blood vessels.

Experiment 1 - Intermittent hyperbaric oxygen exposures effect on hypoxic mice: The graph in Fig. 2 shows the extent of fibroblastic tissue infiltration into the sponge 32 days after implantation. Normoxic

controls achieved an area of $36.3 \pm 3.2 \text{ mm}^2$ infiltration into the sponge. No significant differences were seen in the area of fibroblastic infiltration in hypoxic mice ($35.4 \pm 4.7 \text{ mm}^2$) compared to normoxic controls nor between hypoxic mice receiving intermittent hyperbaric oxygen treatments ($34.7 \pm 4.8 \text{ mm}^2$) and the normoxic controls.

Experiment 2 - EGF's effect on hypoxically conditioned mice: When the sponges were removed from the animals 25 days after implantation, the animals treated with EGF demonstrated slightly increased (NS) areas of fibroblast infiltration compared to placebo. This was true both in animals conditioned to hypoxia for 7 days before and 25 days after sponge implantation ($18.0 \pm 2.5 \text{ mm}^2$ for EGF vs $24.2 \pm 2.5 \text{ mm}^2$ for placebo) and normoxic animals ($20.2 \pm 2.6 \text{ mm}^2$ for EGF vs. $23.8 \pm 1.43 \text{ mm}^2$ for placebo). The increase was consistent in both hypoxic and normoxic groups but the increase was not significantly different. Mice exposed to hypoxic conditioning did not have different areas of fibroblast infiltration from normoxic controls either in the EGF treated or the placebo group (Fig 3).

Experiment 3 - EGF and fibrous encapsulation: The smaller (5 mm diameter) EGF treated sponges implanted in mice exposed to normoxic conditions had a slightly greater (NS) area of fibroblastic tissue infiltration than the placebo group ($3.4 \pm .2 \text{ mm}^2$ for EGF vs $2.9 \pm 0.2 \text{ mm}^2$ for placebo). However, EGF administration significantly ($p \leq 0.05$) increased the area of fibrous encapsulation around the sponge (Fig 4) compared to placebo ($13.0 \pm 2.4 \text{ mm}^2$ for EGF vs $6.8 \pm 2.1 \text{ mm}^2$ for placebo).

Discussion

Using the experimental model employed in this study, hypoxic conditioning did not change the area of fibroblast infiltration into PVA sponges compared to normoxic controls. Although systemic hypoxia conditioning reduced mouse subcutaneous tissue oxygen tension by approximately one-half, the area of fibroblast infiltration into PVA sponges was not altered when compared to controls.

Previous experiments have revealed low tissue oxygen tensions, such as those found in wounds, can have both stimulatory and inhibitory effects on aspects of the wound healing process. Reducing the oxygen tension in tissue culture media from 20% (160 mmHg) to 2.5% (40 mmHg) dramatically increases both the proliferative rate of human diploid fibroblasts in tissue culture and their proliferatory response to EGF and platelet derived growth factor (16). Intermittent exposure of rats to severe systemic hypoxia increased collagen deposition in experimentally produced granulation tissue (17). The previously mentioned experiments provide evidence for hypoxia as stimulants of aspects of the wound healing process, but other studies show high oxygen tensions are necessary for and can stimulate wound healing, while low oxygen tensions inhibit wound repair. The closure of wounds and the formation of scar tissue involve oxygen dependent processes. The secretion of collagen by fibroblasts into the extracellular matrix requires oxygen-dependent enzymatic hydroxylation

of the intracellular precursor of collagen, procollagen (18). The rate of this hydroxylation and subsequent collagen production and secretion by the fibroblast is inhibited by low oxygen tensions and favored by high oxygen tensions (19). In fact, the optimal oxygen tension for collagen production by transformed mouse fibroblasts was found to be 80 mmHg (20). Lowering the oxygen tension can inhibit wound healing. For example, chronic exposure of rats to hypoxic air decreased wound closure rate of full thickness skin wounds (5). Collagen deposition into cellulose sponges in rats was found to be inhibited by hypoxic air and increased by hyperoxic air (6). The differing data found in the literature show that lowering the tissue oxygen tension may have radically different effects on this aspect of the wound healing process.

A possible explanation for these conflicting results may be related to difficulties in actually determining the nature of the cellular microenvironment of cells and their optimal oxygen environment. Low oxygen tensions may be required to activate certain cells in the wound healing process, while the function of other cells may be impaired by low oxygen tensions. As shown in the wound healing module (12), there are zones of actively metabolizing tissue which consume oxygen in regions of variable vascularity due to areas of intense angiogenesis. This creates several zones in the wound healing module where there are widely varying oxygen tensions due to varying metabolic rates and varying amounts of blood perfusion. It is therefore difficult to determine the exact oxygen level to which cells involved in the wound healing process are exposed and

thus predict optimal oxygen tensions for function of these cells.

Non-healing of human wounds is often associated with low oxygen tension in the area of the wound. This low tissue oxygen tension may be a consequence of inadequate blood perfusion, which results in impaired oxygen delivery to the tissue and thus low tissue oxygen tensions, or the low oxygen tension may itself be the primary factor responsible for failure of the wound to heal. Whether impaired closure of wounds is primarily due to inadequate perfusion or to low tissue oxygen tension, there is definitely an association of low oxygen tension with impaired closure of human wounds (21). In this study we suspect that systemically induced hypoxia did not reduce tissue oxygen tensions to a level comparable to that seen in human patients with non-healing wounds. Humans with non-healing wounds may have tissue oxygen tensions as low as 5-10 mmHg (22) whereas we were only able to reduce tissue oxygen tension to the 15-20 mmHg range by reducing ambient oxygen pressure. It may be that in tissue with adequate blood perfusion the lowering of tissue oxygen tension has little effect on the extent of fibroblast infiltration into the PVA sponge or other aspects of the wound healing process.

Human patients with non-healing wounds often undergo hyperbaric oxygen therapy in an effort to correct the tissue hypoxia in the area of the wound and thus promote healing (23). We did not find an effect of intermittent hyperbaric oxygen on the rate of fibroblast infiltration into PVA sponges of hypoxically conditioned mice. This can be explained because the systemic hypoxia did not produce a decrement in fibroblast infiltration.

Hyperbaric oxygen therapy is generally employed to correct the detrimental effect of the tissue hypoxia and not used to stimulate the normal healing process (14). The intermittent hyperbaric oxygen exposures most likely did not affect fibroblast infiltration in this study because the tissue oxygen levels were not limiting the extent of fibroblast infiltration in this experimental model, and thus there was no need for correction of the tissue hypoxia. Although no effect of intermittent hyperbaric oxygen was seen in this study, it is quite possible that intermittent hyperbaric oxygen exposures may have other effects on the wound healing process, such as affecting the density of fibroblastic growth or the amount of collagen deposition in wounds.

Epidermal growth factor increased the rate of fibroblast infiltration into and the area of fibrous encapsulation around the PVA sponge model. The effects of the EGF on fibroblast infiltration were seen in mice maintained in both hypoxic and normoxic environments. Conditioning of the animals to a hypoxic environment did not alter the effect of the EGF on fibroblast infiltration into the sponge.

Epidermal growth factor's effect on fibroblast infiltration and encapsulation of the sponge may be related to its stimulatory effect on cells in the wound healing process. In tissue culture, EGF stimulates fibroblast proliferation as well as migration and proliferation of epidermal cells (24). Epidermal growth factor has also been shown to increase the rate of epithelization in split thickness skin wounds of humans (9). Epidermal growth factor applied in a slow release polymer in this study may have

stimulated the division of fibroblasts which promoted their migration through the trabeculae of the PVA sponge. These cellular effects of EGF on fibroblasts resulted in an increased extent of fibroblast infiltration into the sponge. This increase by EGF on fibroblast infiltration into PVA sponges has been noted previously in this PVA sponge model (25) and others (8).

In this study, the chronic systemic hypoxia did not alter EGF's effect on fibroblast infiltration. The stimulation of the wound healing process by EGF may have increased the metabolic consumption of oxygen by cells, thus removing available oxygen and making the tissue more hypoxic at the wound site. Lowered oxygen tensions did not limit the extent of fibroblast infiltration at the level of stimulation of fibroblast infiltration we achieved with EGF .

The fact that EGF had a more dramatic effect on the size of the fibrous capsule than of the infiltration of fibroblastic tissue into the sponge may relate to the encapsulation of the sponge being a more valid indicator of stimulation or inhibition of wound healing by experimental treatments. This reinforces previous studies which have suggested a need for modification of PVA sponge models (26) to incorporate the examination of parameters besides infiltration into the sponge. The area of encapsulation around PVA sponge implants has not been examined previously because it is difficult to objectively and accurately dissect the fibrous capsule around the sponge from the surrounding tissue. New experimental techniques employing PVA sponges encased in Gore-Tex tubing eliminate this difficulty (27) and may

provide a more reliable and consistent technique for examining the response of experimental animals to PVA sponge implantation.

The results of this study do not support the use of either intermittent hyperbaric oxygen or growth factors as agents which might increase the rate of fibroblast infiltration in this experimental model of wound healing. It is quite possible both intermittent hyperbaric oxygen exposures and EGF may be effective in increasing fibroblast infiltration in hypoxic wounds. More severe hypoxic conditions than those created in our experimental model may be required to fully determine the role of hyperbaric oxygen treatments in severely hypoxic wounds. Epidermal growth factor's increase of the area of the fibrous capsule around the PVA sponge supports it's stimulatory effect on connective tissue growth in this experimental model. New experimental techniques and examination of other aspects of the wound healing process are needed to provide more definitive information concerning the value of growth factor administration and hyperbaric oxygen treatments in the resolution of hypoxic wounds.

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Legends

Fig. 1 Flow diagram of the experimental design.

Fig. 2 Effect of hypoxia (12% oxygen) on fibroblast infiltration into PVA sponges. Hypoxic and hyperoxic exposures were administered between 21-32 days after sponge implantation. Sponges were harvested 32 days after implantation. Vertical bars represent the mean \pm SE. Groups are not significantly different. Values represent mean \pm SE (n = 6-7).

Fig. 3 Effect of EGF on fibroblast infiltration into PVA sponges implanted in animals in hypoxic (12% oxygen) and normoxic (21% oxygen) environments. Animals were exposed to the hypoxia for 7 days prior to and 25 days after sponge implantation. Sponges were harvested 25 days after implantation. Values represent mean \pm SE (n = 6-14).

Fig. 4 Effect of EGF on fibrous infiltration and encapsulation of 5 mm diameter PVA sponge. Sponges were harvested 15 days after implantation. The area of tissue infiltrating the sponge was measured as well as the total area around the sponge encapsulated by fibrous tissue. The area of encapsulation of the EGF treated sponges were significantly different from placebo ($p \leq 0.05$). Values represent mean \pm SE (n = 7).

Figure 1

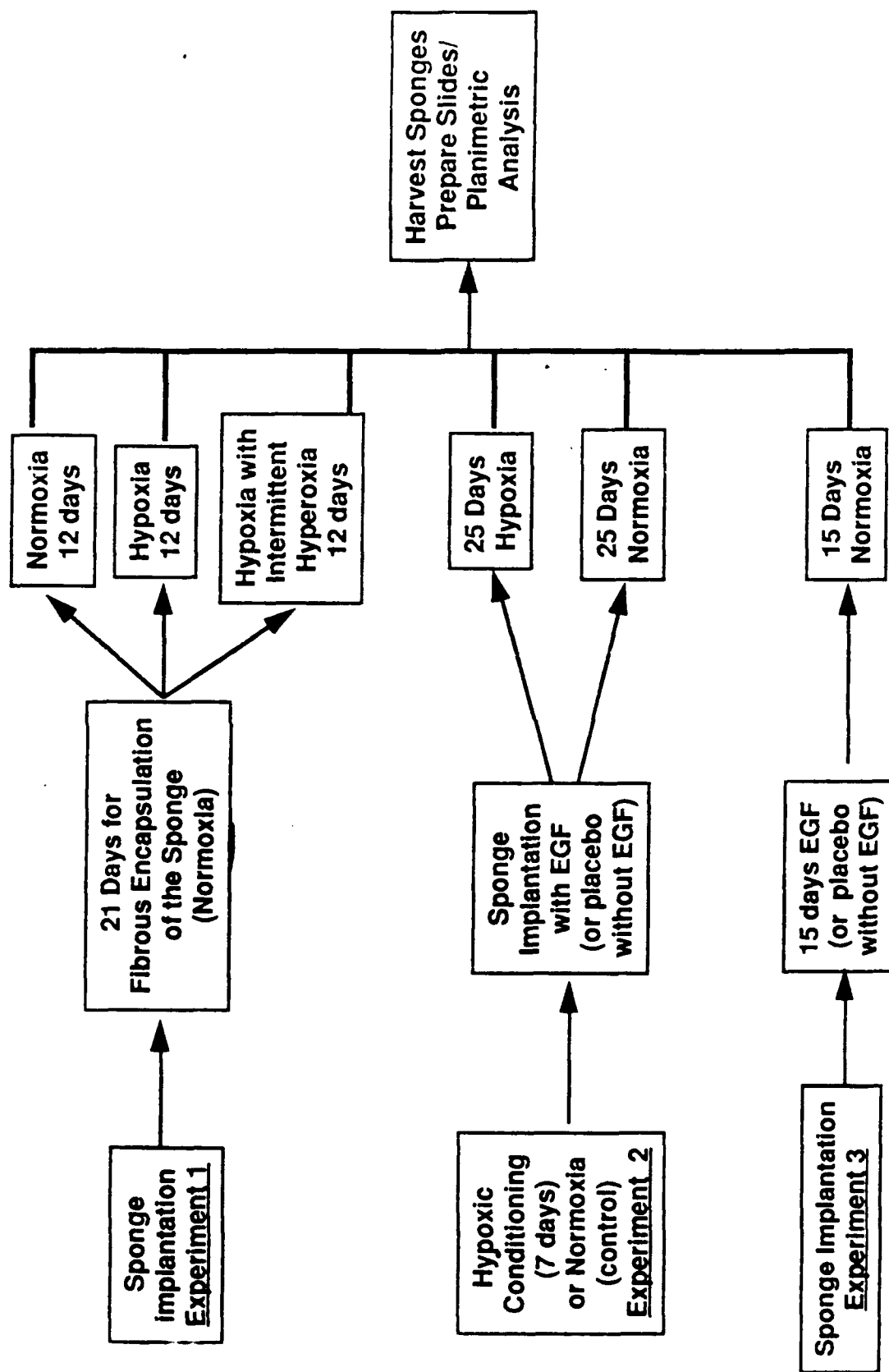


Figure 2

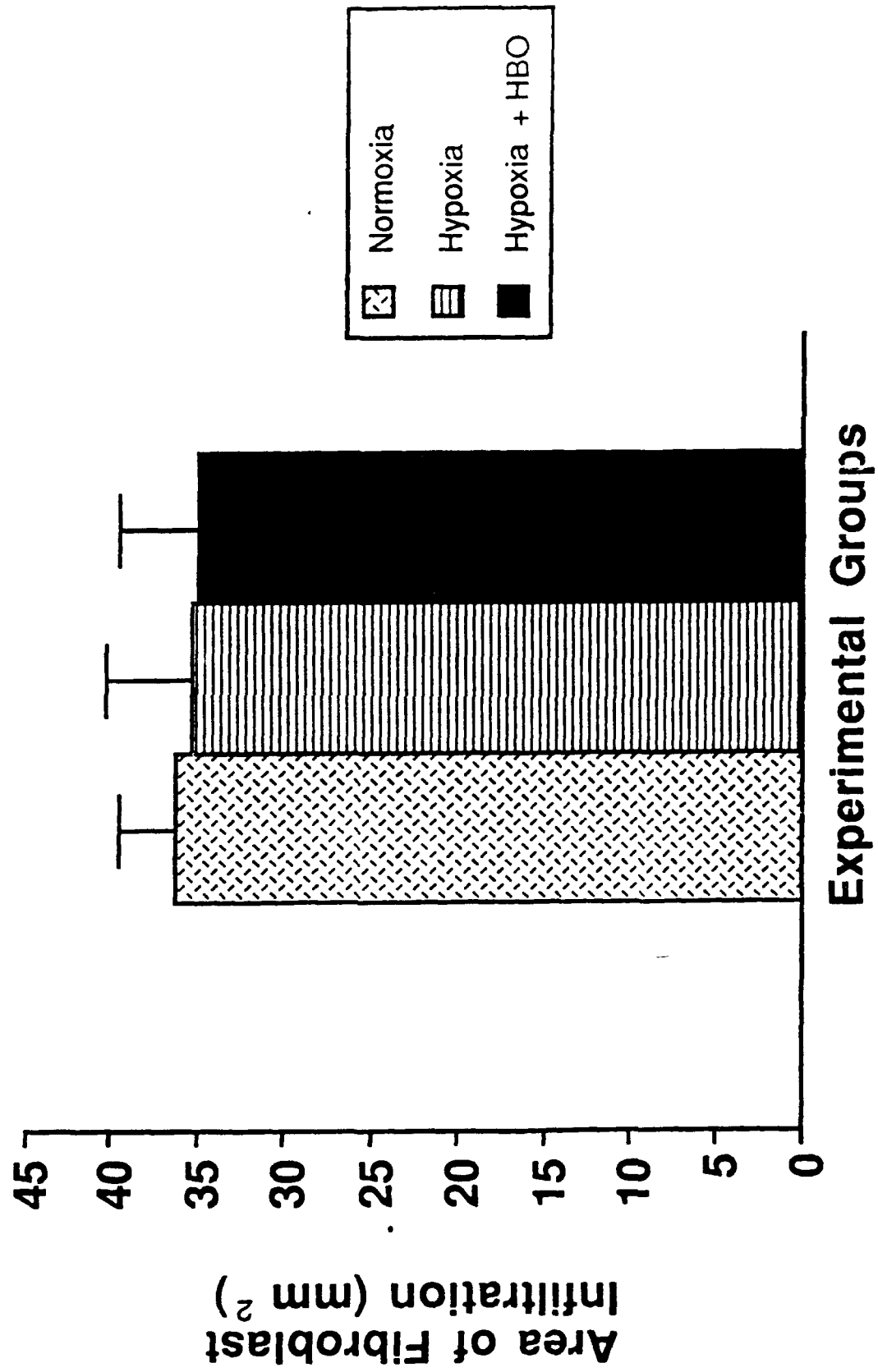


Figure 3

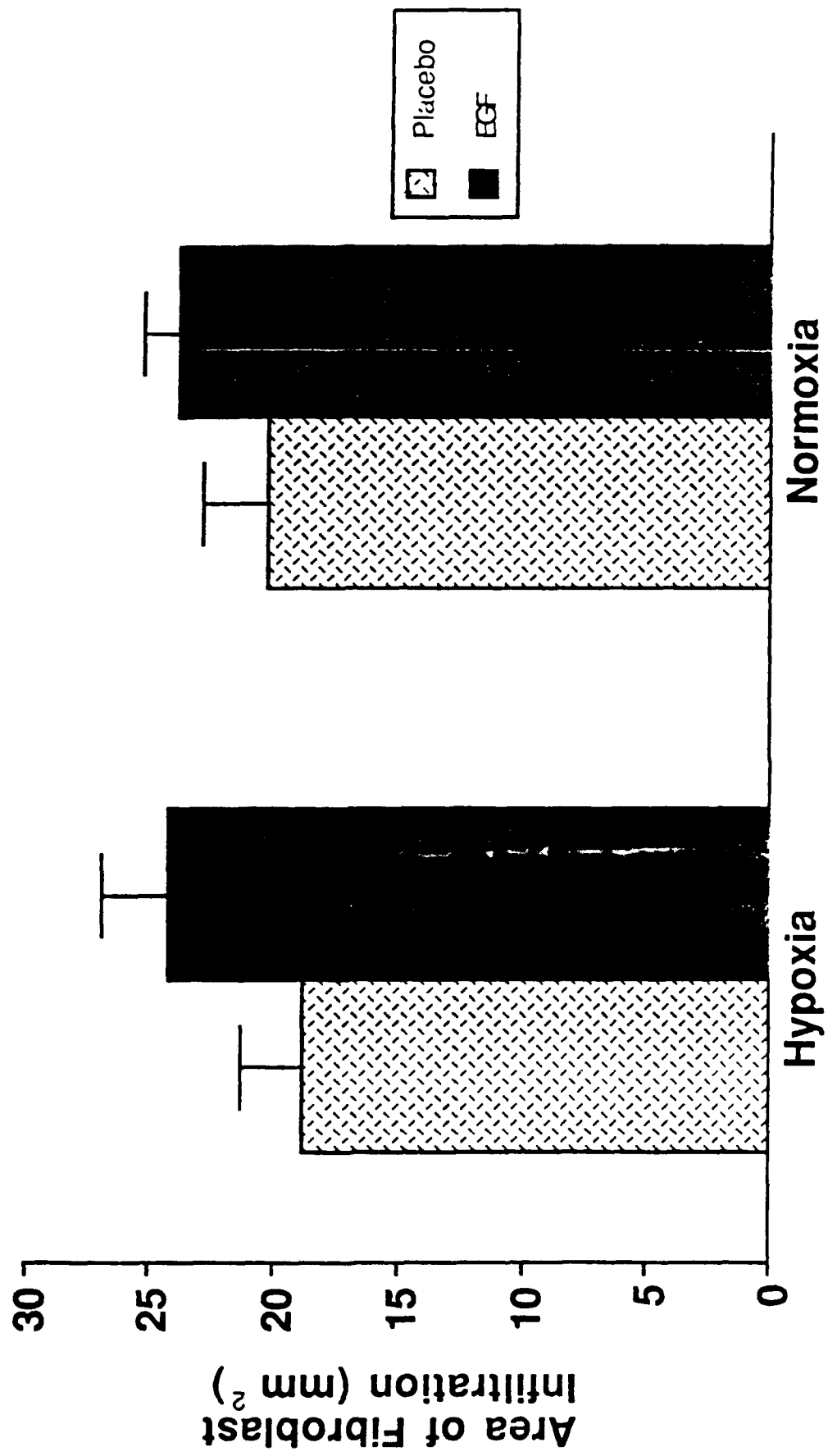


Figure 4

